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Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr ./Patent No./Brevet n°.
D 1910 EP	99112731.7-2116-
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire: Tosoh Corporation	

COMMUNICATION

The European Patent Office herewith transmits as an enclosure the European search report for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

[X] Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

The following specifications given by the applicant have been approved by the Search Division:

[X] abstract [X] title

[ ] The abstract was modified by the Search Division and the definitive text is attached to this communication.

The following figure will be published together with the abstract: NONE

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REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 11 2731

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	ISHIGURO T ET AL: "HOMOGENEOUS QUANTITATIVE ASSAY OF HEPATITIS C VIRUS RNA BY POLYMERASE CHAIN REACTION IN THE PRESENCE OF A FLUORESCENT INTERCALATER" ANALYTICAL BIOCHEMISTRY, vol. 229, no. 2, 10 August 1995 (1995-08-10), pages 207-213, XP000533291 ISSN: 0003-2697 * the whole document *	1-28	C12Q1/68
Y	WO 91 04340 A (CAMBRIDGE BIOTECH CORP) 4 April 1991 (1991-04-04) seee whole doc. esp. claims and figures	1-28	
A	WO 93 22461 A (GEN PROBE INC) 11 November 1993 (1993-11-11) see whole doc. esp. claims		
P,X	EP 0 855 447 A (TOSOH CORP) 29 July 1998 (1998-07-29) * the whole document *	1-28	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)
C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	22 October 1999	Müller, F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO.

EP 99 11 2731

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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22-10-1999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9104340 A	04-04-1991	NONE	
WO 9322461 A	11-11-1993	AU 681082 B	21-08-1997
		AU 4222493 A	29-11-1993
		AU 4523897 A	19-02-1998
		CA 2135073 A	11-11-1993
		EP 0587266 A	16-03-1994
		JP 7506255 T	13-07-1995
		US 5554516 A	10-09-1996
		US 5888729 A	30-03-1999
EP 0855447 A	29-07-1998	JP 10201476 A	04-08-1998

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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Application No.	Ref.	Date
99 112 731.7-2402	D 1910 EP	06.05.2002

Applicant
Tosoh Corporation

Communication pursuant to Article 96(2) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(1) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 78(2) and 83(2) and (4) EPC.

Amendments to the description, claims and drawings are to be filed where appropriate within the said period in **three copies** on separate sheets (Rule 36(1) EPC).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Article 96(3) EPC).

EUROPÄISCHES PATENTAMT - EUROPEAN PATENT OFFICE - OFFICE EUROPEEN DES BREVETS

LEBER T M
Primary Examiner
for the Examining Division

Enclosure(s): 5 page/s reasons (Form 2906)
D3

Registered Letter

The examination is being carried out on the **following application documents**:

Text for the Contracting States:
AT BE CH LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Description, pages:

1-6,8-34,36-38,41, 43,45,48,51,54,57-61, 63-69	as originally filed			
7,7a,35,39,40,42, 44,46,47,49,50,52, 53,55,56,56a,62	as received on	09.11.2001	with letter of	08.11.2001

Claims, No.:

1-27	as received on	09.11.2001	with letter of	08.11.2001

Drawings, sheets:

1/16-16/16	as originally filed

1. General remarks

1.1 The following documents (D) are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D3: Ishiguro T. et al., Nucleic Acid Research, 1996, Vol 24, No 24, pages 4992-4997

This document is cited in the description of the present application and the Applicant referred to it in his letter dated 08.11.2001. A copy is attached to the present communication.

1.2 The amendments filed with letter of 8.11.2001 fulfil the requirements of Art 123(2)

EPC.

1.3 The Applicant provided convincing arguments to overcome the majority of objections raised in the first written communication (dated 17.07.2001). The remaining objections are detailed below.

2. Novelty (Art 54 EPC)

2.1 Novelty is acknowledged for amended claims 1-27 (Art 54 EPC).

3. Inventive step (Art 56 EPC)

3.1 An inventive step is acknowledged for amended claims 1-19 and 23-27 for the same reasons as detailed in the first Official Communication (dated 17.07.2001; see also 4.4 below).

3.2 Claim 20 differs from the closest prior art document D1 in that

- the single or double stranded DNA molecule encompasses an enhancer sequence and in that
- an oligo DNA is added, which is labelled with an intercalative dye that gives a measurable fluorescent signal upon hybridisation.

The technical problem is to provide an improved method for producing a nucleic acid whereby the strength of the promoter is improved and the product specifically detected. The solution referred to in claim 20 is the inclusion of an enhancer sequence and an oligo DNA which is labelled with an intercalative dye.
As far as the enhancer is concerned and in view of the absence of any further arguments provided by the Applicant, the Examining Division maintains the argumentation put forward in its first Official Communication (item 3.3) which resulted in the conclusion that said solution does not form a basis for an inventive step (Art 56 EPC).
With respect to the oligo DNA labelled with an intercalative dye, the Examining Division does not agree with the arguments provided by the Applicant. Document D3 discloses the oligonucleotide YPF-271 which is substituted with the fluorescent intercalating agent YO to form the labelled probe YO-YPF-271 (D3, page 4992 "Material and Methods"). A probe of this results in a clear increase of fluorescence

upon hybridisation with a complementary sequence, be it RNA or DNA (D3, Fig. 4; Fig. 5). D3 moreover discloses fluorescent monitoring of in vitro transcription using said probe (D3, Fig. 6) and also considered the possibility that said labelled probe could itself serve as a primer (D3, page 4992, right column). In conclusion, the arguments raised by the Applicant that in light of D3 the skilled person would not have known whether the labelled oligonucleotides described in D3 might interfere with the amplification reaction and that the skilled would have feared that it hinders the amplification reaction appear to be unconvincing. To the contrary, D3 provides a clear indication for the skilled person to try an oligonucleotide probe labelled with an intercalative dye for detection of an amplification product. In conclusion, the Examining Division maintains its objection to lack of inventive step of claim 20 (Art 56 EPC).

The dependent claims 21 and 22 appear not to add subject-matter which in combination with the features of claim 20 would fulfil the requirements of Art 56 EPC.

4. Other objections

4.1 The description of the present application defines that "The specific nucleic acids sequence means a base sequence within the single-stranded RNA which [...] ends at the 3' end with a sequence complementary to the after-mentioned second single-stranded oligo DNA." It thus appears that claim 1(B) referring to a DNA oligo which is complementary to a sequence **within** the 3'-end of the specific nucleic acid sequence, is unclear and lacks support by the description (Art 84 EPC). The same objection applies to claim 20(A).

4.2 In the first Official Communication, the Examining Division raised the objection that an essential feature, namely a means to cut the DNA/RNA hybrid was missing (item 4.1 B.i). In spite of the arguments provided by the Applicant, the Examining Division maintains said objection (Guidelines C-III 4.2ii) as the method of claim 1, which is defined by the addition of the reagents (A) to (I), requires a means to cut the target RNA in order to release the 5' end of the specific nucleic acid sequence. It appears that there is no support in the description disclosing that the method of claim 1 works even in absence of a cleaving means (Art 84 EPC). The provision of the first single stranded oligonucleotide which binds directly upstream of the 5'

end of the specific nucleic acid sequence (claim 1 (A)) merely "allows" cleavage of the single stranded RNA at the 5'-end of the specific nucleic acid sequence but as such does not represent a means for said cleavage. In this context, claim 6, which refers to a first oligonucleic acid which "is" a ribozyme or DNAzyme appears contradictory to claim 1 (Art 84 EPC) as a ribozyme would not be "complementary" to a target sequence but at best partially be complementary.

4.3 In addition, the only cleaving means which is disclosed in the present application to work with a single stranded oligonucleic acid as referred to in claim 1 appears to be RNAse H. Although, there might be other cleaving means which may work at temperatures up to 60°C, the present application explicitly states that RNAse H becomes inactivated at 60°C (page 19, lines 12-14). Thus, claim 2 referring to a temperature range from 35-60°C lacks support by the description (Art 84 EPC) and the specification reproducibility with respect to this feature (Art 83 EPC).

4.4 Claim 20 should be clarified (Art 84 EPC) in that at least one strand of the double stranded DNA contains the same features as the single stranded DNA (promoter, enhancer, specific sequence) in the correct orientation. It is moreover unclear (Art 84 EPC) how the said method for producing a nucleic acid may work at constant temperature if the target DNA to be amplified is present as double strand. It appears that a step to separate the strand is required for this method to work (Guidelines, C-III 4.2ii).

4.5 Moreover, claim 20 (H) refers to a "fourth single stranded labelled oligo DNA". This is unclear (Art 84 EPC) as there is only one further oligonucleic acid and as there is only one labelled oligonucleic acid.

4.6 The method of claim 20 appears to be suitable for producing a "nucleic acid". This term suggests that both DNA and RNA may be produced. It appears that the method of the present claim 20 starting from a DNA molecule having a promoter, an enhancer and a specific sequence, results in the production of a RNA molecule consisting of the specific sequence (=product 1). Reagent A hybridises to the 3' end of said specific sequence and primes first strand cDNA synthesis (=product 2). The reagent H (labelled oligo DNA) also binds to the specific sequence within the newly produced DNA strand and primes second strand cDNA synthesis

(=product 2). Thus, three products accumulate in the sample. Depending on the positioning of the reagent A and reagent H, the product 1 may be longer than product 2 which in turn may be longer than product 3. In view of the plurality of products that may be obtained, the intended use referred to in claim 20, namely "for producing a nucleic acid" appears to be unclear (Art 84 EPC). It is to note that also in the method of claim 1 a plurality of products can be obtained because of the fact that reagent (I) may also serve as a primer.

5. Concluding remarks

5.1 The Applicant is invited to file new claims which take account of the above comments.

5.2 *If the applicant thinks that all objections raised in this communication are* overcome, then the description should, at the same time, be brought into conformity with the amended claims. Care should be taken during revision not to add subject-matter which extends beyond the content of the application as originally filed (Art 123(2) EPC). Any statements of problems or advantages should be restricted to the letter of reply.

5.3 The amendments should be filed by way of replacement pages, avoiding unnecessary recasting of the description. The Applicant should also take account of the requirements of Rule 36(1) EPC. In particular a fair copy of the amendments should be filed. If handwritten amendments are submitted, they should be clearly legible for the printer.

5.4 In the reply, the parts of the application, as originally filed, which form the basis for the amendments (cf Art 123(2) EPC) should be clearly indicated.

5.5 In order to expedite the procedure, the Applicant is kindly asked to clearly point out where the amendments have been made, possibly by enclosing a copy of the original pages with the corrections in the manuscript.

4992–4997 *Nucleic Acids Research, 1996, Vol. 24, No. 24*

Fluorescence detection of specific sequence of nucleic acids by oxazole yellow-linked oligonucleotides. Homogeneous quantitative monitoring of *in vitro* transcription

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ABSTRACT

We have developed a fluorescent DNA probe, oxazole yellow (YO)-linked oligonucleotide complementary to a target DNA/RNA, which can enhance the fluorescence on hybridizing with a target nucleotide. We demonstrated the applicability of the YO-linked oligonucleotide probe to real-time monitoring of the *in vitro* transcription process of a plasmid DNA constructed containing the 5′-terminus non-coded region of hepatitis C virus RNA. In the process of *in vitro* transcription in the presence of YO-linked complementary oligonucleotide, the fluorescence of the reaction mixture showed a time-dependent linear increase corresponding to the generated target RNA product.

INTRODUCTION

An easy and simple method has been required to detect specific base sequences of nucleic acids in clinical diagnostics or molecular pathology as well as for the elucidation of various biochemical processes associated with the expression and transmission of genetic information (1,2).

Most of the current standard methods require hybridization on solid supports, electrophoretic migration, solid-phase capture or HPLC separation for the detection of the specific nucleic acid sequences (3). These separation steps are tedious and time-consuming in a clinical setting and, in particular, can be one of the main obstacles for the application of PCR-based assays in laboratory diagnosis. It is necessary to control the contamination of PCR products in post-PCR processes, such as sampling PCR products, to apply them to gel electrophoresis (4–6).

A homogeneous format can be a strategy to relieve the obstacles due to those separation process in practice and so far many researchers have challenged the development on several approaches (7–8). Helene and co-workers reported the fluorescent detection of a double-stranded DNA in forming triple helix with donor- and acceptor-linked DNA probes in terms of their energy transfer (9). Sixou recently demonstrated fluorescent detection of intracellular oligonucleotide hybridization in living cells by fluorescence energy transfer (10).

It is known that a DNA intercalator, oxazole yellow (YO), shows marked enhancement of fluorescence on binding to double-stranded DNA while YO itself is virtually non-fluorescent in the absence of DNA (11). Recently, we were successful in the fluorescence monitoring of the polymerase chain reaction by adding fluorescent DNA intercalator, oxazole yellow, in the reaction mixture and the quantification of starting number of a target template without any post-PCR analysis such as electrophoresis of PCR products (12).

We attempted to design a fluorescent DNA probe to enable us to construct an easy and specific homogeneous method to detect a nucleic acid sequence. We report herein the property of YO-linked oligonucleotide, which can emit enhanced fluorescence by binding to a complementary oligomer, and its application to *in situ* fluorescence detection of a specific sequence of RNA produced by *in vitro* transcription.

MATERIALS AND METHODS

Preparation of YO linked DNA probe

Thiol-modified oligonucleotide 5′-AAAAA*AAAAAAAA-3′ (named DAL-13) and 5′-CTCGC*GGGGGCTG-3′ (named YPF-271), were supplied from Yuki Gosei Yakuhin Kogyo, Co. Ltd, Japan, in which A*and C* are adenine and cytidine having a -$(CH_2)_2NHCO(CH_2)_2SH$ appendage at 3′ phosphorus, respectively. The nucleic acid sequence, 5′-CTCGC*GGGGGC-3′, of YPF-271 is complementary to the positions 223–233 of the 5′-terminus non-coded region of hepatitis C virus RNA (HCV 5′NCR) (13). Two bases from the 3′ terminus of YPF-271, TG, are intentionally mismatched with the target RNA to avoid the extension by a polymerase.

Oligo dT (30mer) and oligo dA (30mer) were purchased from Yuki Gosei Yakuhin Kogyo, Co. Ltd, Japan.

Synthetic oligonucleotides, complementary to YPF-271, were purchased from Yuki Gosei Yakuhin Kogyo, Co. Ltd, Japan, for DNA (named YPF-271+D), and from TaKaRa, Japan, for RNA (named YPF-271+R). Sequences of each oligomer are as follows: YPF-271+D: 5′-GTGCCCCCGCGAG-3′, and YPF-271+R: 5′-GUGCCCCCGCGAG-3′.

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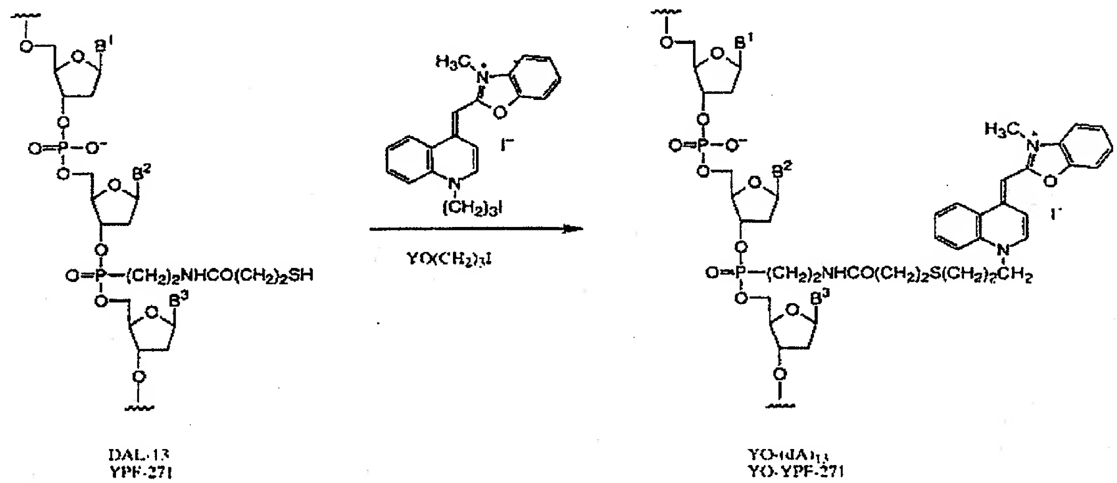

Figure 1. Synthesis of YO-linked oligonucleotide probe. The details are described in the text.

YO-(dA)13. (Fig. 1) Aqueous dithiothreitol (0.01 M, 20 µl) was added to the fraction containing purified DAL-13 by HPLC and shaken by a vortex mixer (solution A). $YO(CH_2)_3I$ (14) was saturated in a mixture of DMF (200 µl), 1.0 M phosphate buffer (pH 10.0, 300 µl), and H_2O (500 µl) under argon (solution B). Solutions A and B were mixed under strictly unaerated argon atmosphere (solution A:solution B = 1:2 ~ 1:3). The mixture stood for 2 h and was purified by Sephadex G-25 (5% acetonitrile/0.1 M triethylamine–acetic acid, pH 7.0). The purified fraction was concentrated *in vacuo*, and dried. The resultant was dissolved by distilled water, and further purified by HPLC (column: VX-Nucleotide, Shinwa Chemical, Japan/linear gradient mode from buffer (a) to buffer (b) [(a) 5% acetonitrile, (b) 50% acetonitrile, both contain 0.1 M triethylamine–acetic acid (pH 7.0)]. The fraction obtained was also concentrated *in vacuo* and dried to remove triethylamine–acetic acid thoroughly so as to give the YO derivative of DAL-13 [named $YO\text{-}(dA)_{13}$] finally.

YO-YPF-271. Thiol-modified oligonucleotide (YPF-271) was reacted with $YO(CH_2)_3I$ and purified, according to the same procedure as described above, to give the corresponding YO derivative (named YO-YPF-271).

DNA fragment bearing RNA polymerase promoter region

We constructed a plasmid DNA SKP/SR1-P2-6 which contains a T7 promoter, *Xho*I site, HCV 5′ noncoding region (290 bp), *Hin*dIII site and T3 promoter in the order and orientation shown in Figure 2. The details of the construction of the plasmid were described in our previous report (12).

SKP/SR1-P2-6 was digested with *Hin*dIII and *Xho*I to result in linear DNAs of HCV 5′ NCR sequence containing T7 promoter and T3 promoter, respectively, followed by purification with HPLC.

Hybridization and fluorescence measurement

Each of dT (30mer) and dA (30mer), final concentration 5 nM, was added into a hybridization buffer of 40 mM Tris–HCl, pH 8.0, containing dithiothreitol (5 mM), $MgCl_2$ (8 mM), BSA (50 µg/ml) and $YO\text{-}(dA)_{13}$ (5 nM).

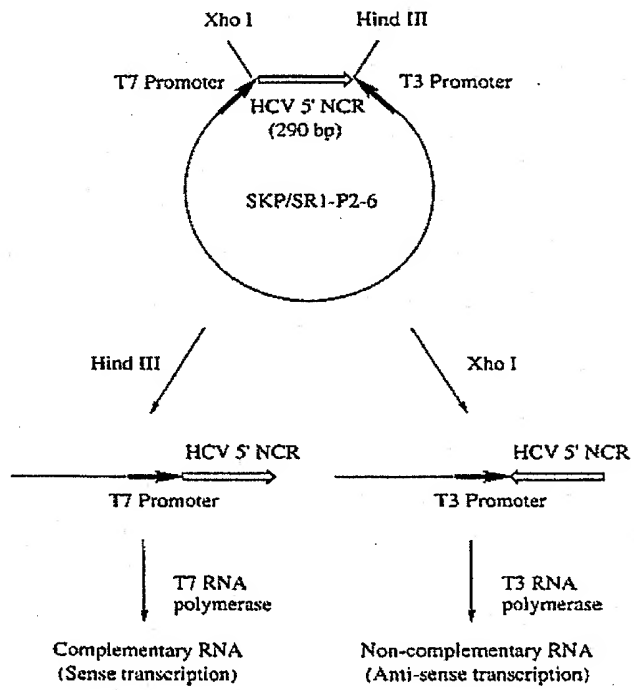

Figure 2. Plasmid DNA SKP/SR1-P2-6 contains HCV 5′ NCR sequences (290 bp).

Each of YPF-271+D and YPF-271+R, final concentration 50 nM, was added into a hybridization buffer (1× SSC, 1 mM EDTA), containing YO-YPF-271 (50 nM).

A volume of 500 µl of the resultant mixture was transferred into a quartz cuvette (1 × 1 × 4.4 cm, 3.5 ml) in temperature control module supplied with the spectrometer, model FP-777, followed by the measurement of the fluorescence spectra (excitation wavelength 490 nm/HW 5 nm, fluorescence spectrometer model FP-777, Jasco, Japan).

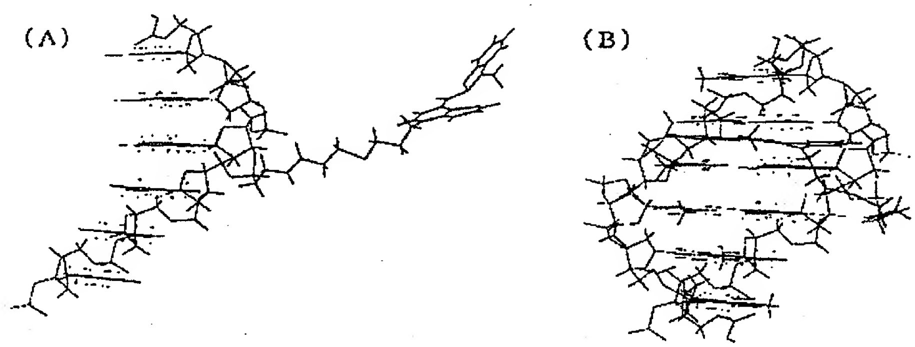

Figure 3. Models of YO-linked oligonucleotide (A) and the hybridized complex with oligo dT (B), calculated and drawn by HyperChem Nucleic Acids Databases (Hypercube, Inc., Canada). The bold lines indicate YO moiety.

Melting temperature

Each of YPF-271 and YO-YPF-271, final concentration 1.5 μM, was added into a hybridization buffer (1× SSC, 1 mM EDTA, pH 7.0), containing a synthetic complementary sequences as a target (YPF-271+D: 5′-GTGCCCCCGCGAG-3′).

T_m values were obtained as the temperature of half dissociation on the basis of the measurement of the hyperchromic effect of UV absorption at 260 nm (UV spectrometer model U-2000, Hitachi, Japan).

In vitro transcription

In vitro transcription of SKP/SR1-P2-6/*Hin*dIII and SKP/SR1-P2-6/*Xho*I were performed by T7 (TaKaRa, Japan) or T3 (Toyobo, Japan) RNA polymerase (0.1 U/μl), respectively, in a transcription buffer of 40 mM Tris–HCl, pH 8.0, containing dithiothreitol (5 mM), $MgCl_2$ (8 mM), rNTP (0.4 mM), template DNA (0.015 mg/ml), YO-YPF-271 (0.025 pmol/μl), RNase inhibitor (2 U/μl).

As shown in Figure 2, the resultant of the digestion with *Hin*dIII (SKP/SR1-P2-6/*Hin*dIII) affords to produce RNA complementary to YO-YPF-271 by *in vitro* transcription using T7 RNA polymerase. On the other hand, *in vitro* transcription of the digested fragment with *Xho*I (SKP/SR1-P2-6/*Xho*I) using T3 RNA polymerase does not produce RNA complementary to YO-YPF-271.

The present concentration of the template, 0.015 mg/ml, corresponds to $\sim 7\times10^{-6}$ nmol/μl, or 4×10^{9} copies/μl. SKP/SR1-P2-6 contains 3251 bp, 2961 bp + 290 bp, of which the molecular weight is estimated to be 2.1×10^{6} (660 × 3251).

The reaction was carried out in the quartz cuvette in 500 μl of the transcription buffer at 40°C to be monitored the fluorescence intensity with the fluorescence spectrometer. The sample was excited at 490 nm and the fluorescence emission was measured at 510 nm (HW: 5 nm).

The reaction was also performed in 700 μl of the transcription buffer in measuring the fluorescence intensity (ex: 490 nm, em: 510 nm, HW: 10 nm). Aliquots of 16 μl of the reaction solution were collected from the cuvette at 5 min intervals to be applied to gel electrophoresis to quantify the product with a densitometer (ACI Image Analysis System, ACI Japan).

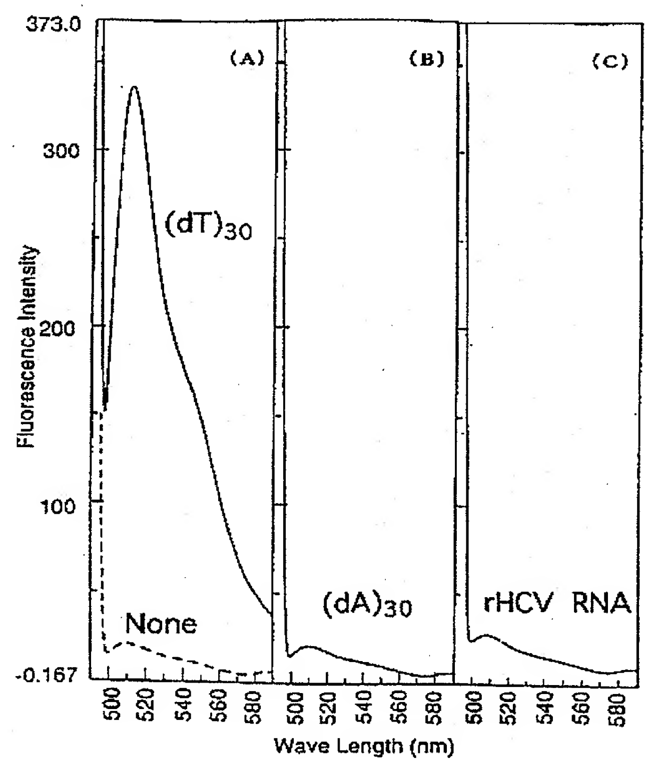

Figure 4. Fluorescence spectra (excitation at 490 nm) of YO-linked oligo dA probe in presence of oligo dT 30mer (A), dA 30mer (B) and recombinant HCV RNA (C), respectively. In (A), the broken curves indicate the fluorescence spectra of YO-linked oligo dA alone.

RESULTS AND DISCUSSION

Fluorescence enhancement of the YO-linked DNA probe by hybridization

It is generally known that some DNA intercalators bind double-stranded DNA to enhance the fluorescence. Oxazole yellow (YO), one of the fluorescent DNA intercalative dyes, shows marked enhancement of the fluorescence on binding to a double stranded

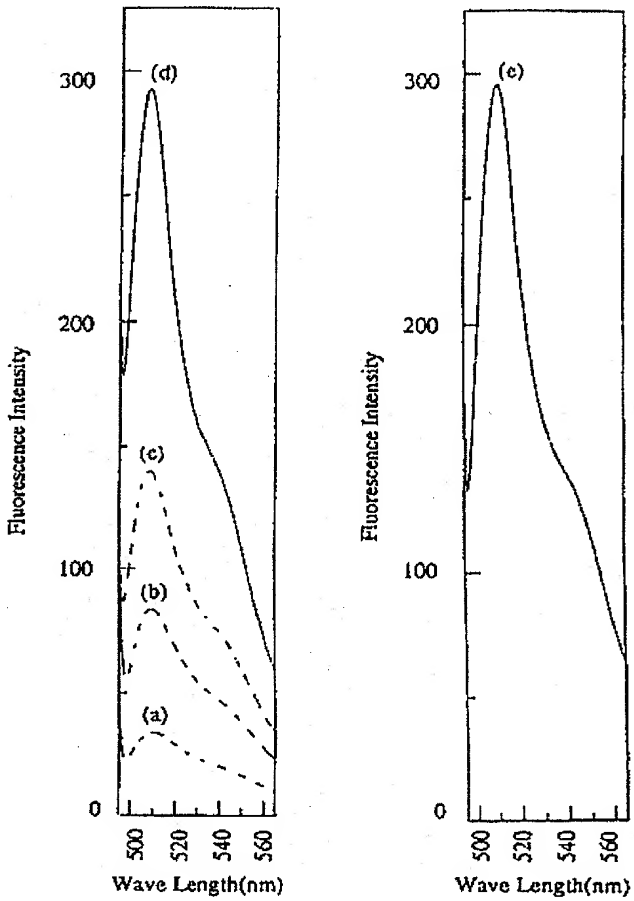

Figure 5. Fluorescence spectra (excitation at 490 nm) of YO-YPF-271; (a) at 70°C, (b) at 50°C, (c) at 25°C, (d) in the presence of complementary DNA, YPF-271+D, at 25°C and (e) in the presence of complementary RNA, YPF-271+R, at 25°C.

DNA (11,12). It is, therefore, thought that an oligonucleotide equipped with a YO would emit enhanced fluorescence on binding to a complementary oligomer.

We designed a YO-linked oligonucleotide, YO-$(dA)_{13}$, 5′-AAA-AA*AAAAAAAA-3′, in which A* is adenine having YO through a linker, -$(CH_2)_2$NHCO$(CH_2)_2$S-YO, appendage at 3′ phosphorus. As shown in Figure 3, the model of the hybridized complex with oligoT suggests that the linker of YO-$(dA)_{13}$ is readily to deliver the YO to adjacent base pairs of formed double-stranded DNA with the complementary oligo T. The hybridization, thus, would bring the enhanced fluorescence.

In Figure 4, the presence of 30mer brought marked enhancement of the fluorescence of YO-$(dA)_{13}$ while YO-$(dA)_{13}$ itself is virtually non-fluorescent. Non-complementary nucleic acids, dA 30mer and rHCV RNA, did not give the fluorescent enhancement of YO-$(dA)_{13}$ in the mixture.

The present results imply that YO-$(dA)_{13}$ recognizes the complementary nucleic acids to hybridize with it and YO moiety intercalates into formed double-stranded DNA so as to enhance the fluorescence, as YO itself showed in dye–DNA intercalation complexes.

Fluorescence monitoring of *in vitro* transcription

YO-linked DNA probe described in the present work would be applicable to homogeneous fluorescence detection and quantification of specific nucleic acids.

We chose hepatitis C virus (HCV) RNA as our target sequence for the detection by a YO linked DNA probe.

We designed a YO linked DNA probe, YO-YPF-271, complementary to the 5′-terminus non-coded region of hepatitis C virus RNA (HCV 5′NCR). A YO moiety was inserted at an internal cytidine, C*, of a 13mer, 5′-CTCGC*GGGGGCTG-3′.

We investigated the property of YO-YPF-271 in forming duplex on a target DNA or RNA (Fig. 5).

YO-YPF-271 showed the enhancement of the fluorescence in the presence of the complementary deoxynucleotide sequences (Fig. 5c and d).

It is noted that the fluorescence intensity of YO-YPF-271 itself decreases remarkably with the increase of the temperature of the solution (Fig. 5a–c). The intrinsic fluorescence signal is thought to be caused mainly from the formation of the secondary structure of YO-YPF-271 itself, or self-assembling in the solution, and the intercalation of the YO moiety into the double-stranded domain, while YO-$(dA)_{13}$ itself cannot form such a secondary structure so

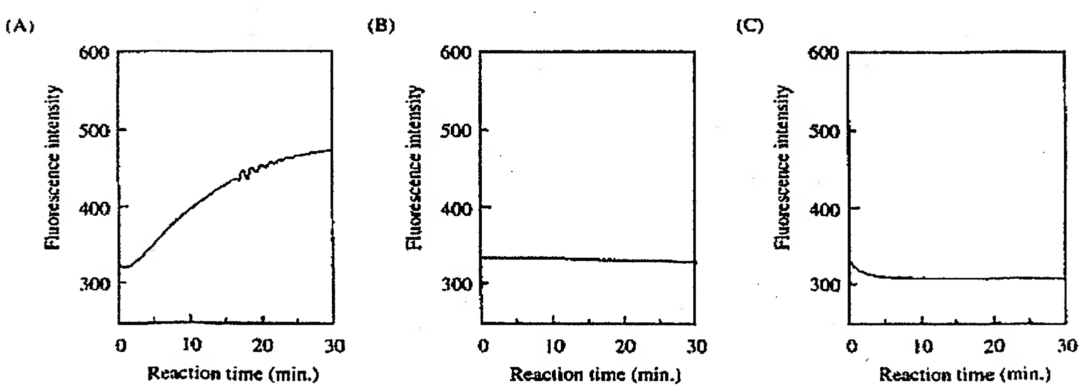

Figure 6. Fluorescence monitoring of *in vitro* transcription of SKP/SR1-P2-6 in the presence of YO-YPF-271 (0.025 pmol/l). (A) Transcription with T7 RNA polymerase on digested fragment of SKP/SR1-P2-6 with *Hin*dIII; (B) digested fragment of SKP/SR1-P2-6 with *Hin*dIII omitting T7 RNA polymerase and (C) transcription with T7 RNA polymerase on digested fragment of SKP/SR1-P2-6 with *Xho*I.

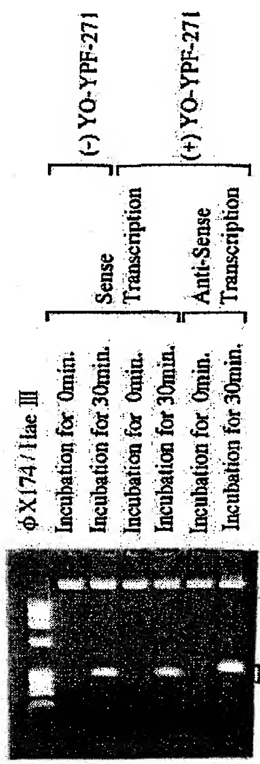

Figure 7. Gel electrophoresis confirming the production of both the complementary RNA (third lane from the right) and the non-complementary RNA (first lane from the right) in the presence of YO-YPF-271 (0.025 pmol/l) (2% agarose gels stained with 0.5 µg/ml of ethidium bromide). The transcription in the absence of YO-YPF-271 are shown in the third lane from the left.

as to show marked enhancement of the fluorescence on binding to the target oligo T, as well as lower intensity in the target's absence.

Figure 5d and e also reveals that YO-YPF-271 gives the fluorescence enhancement for RNA target as well as for DNA. Marked difference of the enhancement was not found in each case.

Therefore, we carried out *in vitro* transcription in the presence of YO-YPF-271 and monitored the fluorescence intensity of the reaction mixture in the time course (Fig. 6). When the *Hin*dIII digestion product of SKP/SR1-P2-6 was subjected to *in vitro* transcription using T7 RNA polymerase, a time-dependent linear increase of the fluorescence intensity was observed (Fig. 6A). In contrast, the fluorescence was not increased in the case of the transcription of the *Xho*I product of SKP/SR1-P2-6 with T3 RNA polymerase (Fig. 6C), indicating that non-complementary RNA was not detected by YO-YPF-271. The same reaction without using T7 RNA polymerase resulted in no increase of fluorescence because RNA was not produced (Fig. 6B). In Figure 7, gel electrophoresis of the products at 30 min incubation shows the production of both the complementary RNA in antisense transcription and the non-complementary RNA in sense transcription.

In addition, there is no difference in the yield found between transcription in the presence or absence of YO-YPF-271 (Fig. 7). The present results indicate that YO-YPF-271 specifically binds the target RNA in the course of the reaction to give rise to the fluorescence enhancement, and the presence of YO-YPF-271 in the *in vitro* transcription has no effect on the activity of RNA polymerase.

We assessed further the relationship between the increase of the fluorescence and the production of RNA. In the course of transcription, aliquots were collected at 5 min intervals to be applied to gel electrophoresis to quantify the product with a densitometer, in measuring the fluorescence intensity of the reaction mixture (Fig. 8). The amount of the RNA product also increased time-dependently corresponding to the enhancement of the fluorescence of the reaction mixture. The yield of RNA after 30 min incubation reached 0.3 pmol/µl, as shown in Figure 8C. One unit of the activity of T7 RNA polymerase used in our present work is specified in manufacturer's data as the capability of uptake of 1 nmol of GMP in 1 h incubation at 37°C to lead to

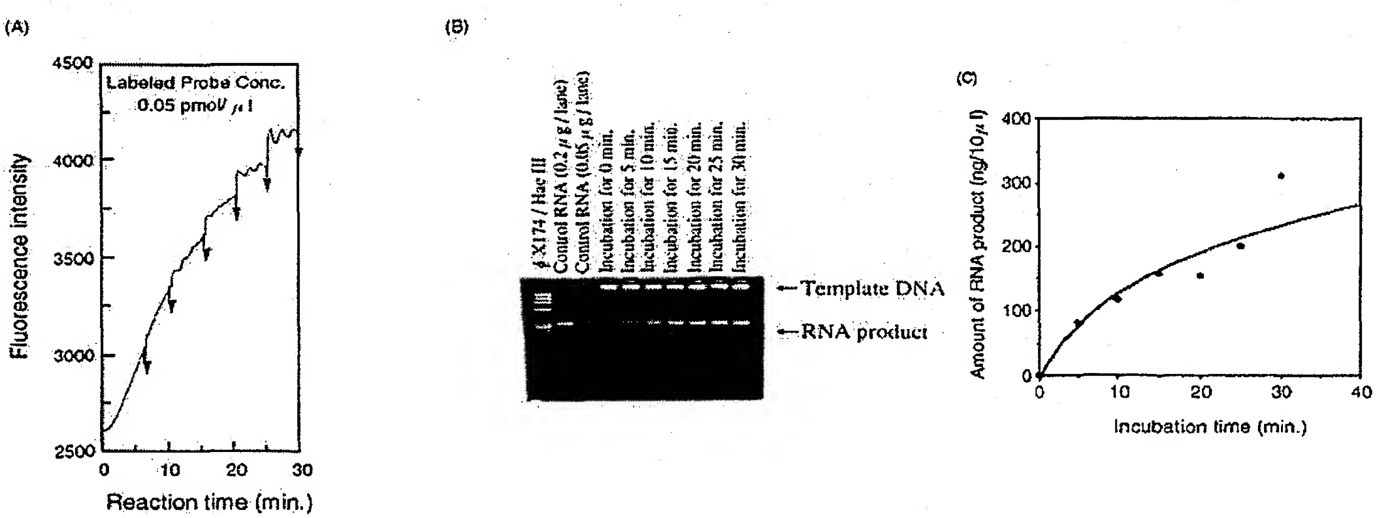

Figure 8. Quantification of the *in vitro* transcription of SKP/SR1-P2-6 under T7 promoter (YO-YPF-271: 0.025 pmol/l). (A) Increase of the fluorescence of the transcription reaction mixture. Aliquots were collected every 5 min shown by arrows. (B) Agarose gel electrophoresis of each aliquot (2% agarose gels stained with 0.5 µg/ml of ethidium bromide). (C) Plot of the RNA content in each aliquot.

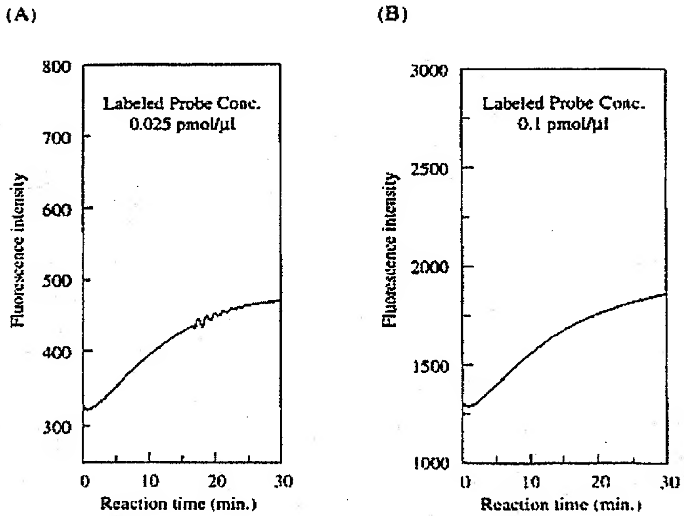

Figure 9. Influence of the concentration of YO-YPF-271 on the detection of *in vitro* transcription. (A) 0.025 pmol/ml; (B) 0.1 pmol/ml.

the estimation of the yield, 0.2 pmol/µl (1 nmol × 0.1 U/µl × 0.5 h/290 bases), which corresponds well with the above experimental value. The CV of the fluorescence increase was ~8%.

On the basis of the study demonstrated herein, it is said that increase of the fluorescence intensity corresponds to the RNA products of the transcription, and the rate of the increase of the fluorescence intensity can be a marker for the detection and quantification of target nucleic acid sequences with excellent reproducibility.

In the fluorescence profile of monitoring the *in vitro* transcription by 0.025 pmol/ml (Fig. 9A) and 0.1 pmol/ml (Fig. 9B) of YO-YPF-271, the increase of the fluorescence intensity at 30 min on the initial fluorescence intensity, F(30 min) – F(0 min), was 570 and 150, respectively. The former is mostly four times higher than the latter, corresponding to the ratio of the amount of added probe in the reaction mixture. In other words, most of the added probe in the initial reaction mixture was consumed to form the complex with the product RNA in the transcription.

To evaluate the affinity of the probe in question, we investigated the melting temperature of the YO-linked oligo DNA probe-target oligo DNA complex (15). According to the report of Helene and co-workers (16), T_m of the intercalator-labeled oligomer tends to be higher than the unmodified one. In our experimental conditions, the YO-linked oligo DNA probe-target oligo DNA complex ($T_m = 66°C$) was also slightly more stable than the corresponding unmodified duplex ($T_m = 63°C$).

Those results imply that rapid equilibrium is established for the formation of the probe–target complex under the present incubation temperature (40°C) so as to ensure the capability of the probe to monitor the dynamic process, such as the production of target RNA in the course of the transcription.

On the basis of the work described here, the authors can readily suggest that the oxazole yellow (YO)-linked oligonucleotide probe presented herein brings a specific and simple homogeneous strategy for fluorescence detection and quantification of a target nucleic acid sequence without any separation steps such as electrophoresis.

The present success of the applicability of the probe to real-time monitoring of the *in vitro* transcription showed that YO-linked DNA probe can be a powerful and versatile tool with which to construct a new methodology to study the dynamics of gene expression, and also to provide a more practical way of detecting and quantifying a target sequence in a clinical specimen specifically in a homogeneous format.

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054

TITLE: DESIGN OF RIBONUCLEASE MIMICS FOR SEQUENCE-**SPECIFIC CLEAVAGE** OF **RNA**

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ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Hydrolysis of RNA by imidazole conjugates capable of binding to RNA have been investigated. Spermine-imidazole conjugate in the presence of imidazole buffer **cleaves RNA** at the **single-stranded** pyrimidine-purine sequences. Oligonucleotides with a diimidazole construction at the terminal phosphate **cleave** tRNA target in vicinity of the complementary sequences.

specific cleavage of **RNA** in a duplex.

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CORPORATE SOURCE: Central Research Laboratories, Ciba, Basel, Switzerland.

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Entered Medline: 19961107

AB Lanthanide complexes covalently attached to oligonucleotides have been shown to **cleave** RNA in a sequence-specific manner. Efficient **cleavage**, however, is at present limited to **single-stranded RNA** regions, as **RNA** in a duplex is considerably more resistant to strand scission. To overcome this limitation, we have designed and synthesised artificial nucleases comprising lanthanide complexes covalently linked to oligodeoxyribonucleotides which **cleave** a partially complementary RNA at a bulged site, in the duplex region. Strand scission occurs at or near the bulge. **Cleavage** of the RNA target by the metal complex can be addressed via the major or the minor groove. In an example of a competitive situation, where the **cleavage** moiety has access to both a bulge and a single-strand region, transesterification at the bulge is favoured. Such artificial ribonucleases may find application as antisense agents and as tools in molecular biology. In addition, the results may have importance for the design of artificial ribonucleases which are able to act with catalytic turnover.

Antisense oligonucleotide containing an internal, non-nucleotide-based linker promote site-**specific** **cleavage** of **RNA**.

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AB We have designed and synthesized a series of novel antisense methylphosphonate oligonucleotide (MPO) **cleaving** agents that promote site-specific **cleavage** on a complementary RNA target. These MPOs contain a non- nucleotide-based linking moiety near the middle of the sequence in place of one of the nucleotide bases. The region surrounding the unpaired base on the RNA strand (i.e. the one directly opposite the non-nucleotide-linker) is sensitive to hydrolytic **cleavage** catalyzed by ethylenediamine hydrochloride. Furthermore, the regions of the RNA comprising hydrogen bonded domains are resistant to **cleavage** compared with **single-stranded** **RNA** alone. Several catalytic moieties capable of supporting acid/base hydrolysis were coupled to the non-nucleotide-based linker via simple aqueous coupling chemistries. When tethered to the MPO in this manner these moieties are shown to catalyze site-specific **cleavage** on the RNA target without any additional catalyst.